

MALDI-TOF-MS AS A RAPID AND SPECIFIC METHOD FOR THE IDENTIFICATION OF *BACILLUS* PATHOGENS IMPLICATED IN BIODEFENSE

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INTRODUCTION

The genus *Bacillus*, commonly referred to as the aerobic spore-bearing bacilli comprise several species that are poorly circumscribed and consequently present considerable difficulties for accurate identification (1-4). Some such as *B. cereus* are important foodborne pathogens while several species including *B.alvei*, *B. megaterium*, *B. coagulans*, *B. laterosporus*, *B. subtilis*, *B. sphaericus*, *B. circulans*, *B. brevis*, *B. licheniformis*, *B. macerans*, *B. pumilus* and *B. thuringiensis* are emerging as important human pathogens. At present, *B. anthracis*, the causative agent of anthrax, has been the focus of much attention as a potential agent for bioterrorism. Current methods of identifying species include biochemical tests, serology, long-chain cellular fatty acid analysis and more recently DNA sequence-based methods. However, because of the high degree of sequence homology between some species, a polyphasic approach which includes other rapid methods are being actively pursued. The aim of the present study is to investigate the potential of Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) as a rapid and specific method, "Bacterial Mass Fingerprinting" (Figures 1 and 2), for the identification of *Bacillus* species. Once this is established, the standard operating procedures devised from this study will be used to investigate the potential of this technique for the rapid delineation of *B. anthracis* isolates.

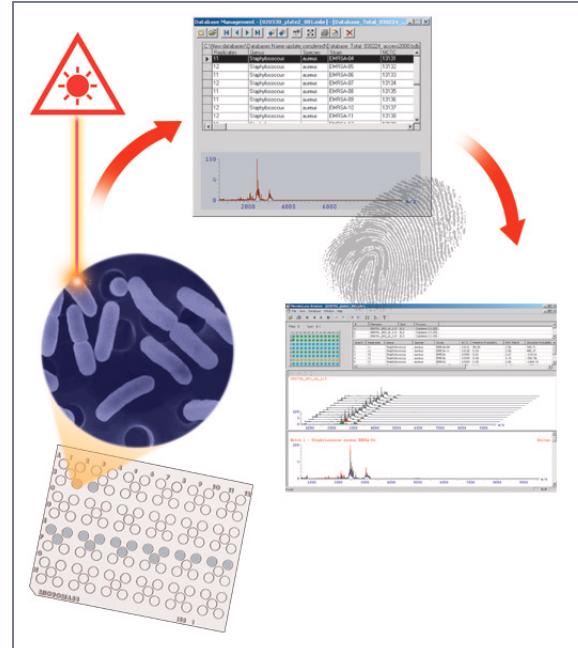


Figure 1. Bacterial Mass Fingerprinting applies proven biopolymer mass spectrometry techniques to the analysis of intact bacteria. The method allows the diagnostic population of macromolecules expressed on the surface of bacteria to be rapidly characterized by molecular weight. The resulting mass spectrum provides a unique physico-chemical fingerprint for the species tested. Mass fingerprints of unknowns can be reliably matched against databases of reference mass fingerprints.



Figure 2. Sample preparation is quick and easy—intact cells from primary culture are smeared across a stainless steel target plate and allowed to co-crystallize with a UV-absorbing matrix. After drying, the target is placed into the MALDI-TOF mass analyzer.

METHODS AND MATERIALS

Bacterial Isolates and Culture Conditions:

The current study is part of a larger ongoing programme to develop a more comprehensive database for microbial identification which now comprises some 2,200 spectral entries (Database release, March 2003). Methods were as described previously (6-11). For the present study, 108 validated UK National Collection of Type Culture *Bacillus* strains encompassing 15 different species were included while unknown strains, sent into the HPA for identification were tested against the database. The NCTC reference strains were *Bacillus "freudenreichii"*, *B. atrophaeus*, *B. badius*, *B. cereus* (13 strains), *B. circulans* (5 strains), *B. coagulans* (5 strains), *B. firmus*, *B. aterosporus* (3 strains), *B. licheniformis* (16 strains), *B. megaterium* (8), *B. mycoides* (5 strains), *B. pumilus* (7 strains), *B. sphaericus* (9 strains),

B. subtilis (12 strains) and 21 strains of various poorly classified bacilli.

1. Database

All the NCTC strains for the database were prepared from freeze-dried ampoules as follows:

- Reconstitution of ampoules in accordance with NCTC guidelines, 'Opening of Ampoules'¹⁵.
- Incubation generally 24 hours at 37 °C on Columbia Blood agar (CBA, Oxoid, Basingstoke, UK) containing 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks. UK.), as supplied by the Public Health Laboratory Service accredited laboratories, in anaerobic atmosphere.
- Two further sub-cultures prior to MALDI-TOF-MS analysis.

2. NCTC Test Strains and Clinical Isolates

Prepared as above either from ampoules or from nutrient agar slopes & analyzed against the database (release 2003) to find the top 8 best matches.

Preparation for MALDI-TOF-MS analysis of NCTC strains and Clinical isolates

- Using a 1 µL culture loop, several bacterial colonies were applied to 12 target plate wells (twelve wells per strain).
- Samples were air-dried for at least 1 hour.
- Overlaid with 1 µL of matrix solution: 5-chloro-2-mercaptopbenzothiazole (Sigma-Aldrich Chemical Company) in Acetonitrile: Methanol: Water (1:1:1) with 0.1% (v/v) formic acid and 0.01M 18-crown-6.
- Allowed to air dry.

INSTRUMENTATION

Analysis was performed using a Waters® Micromass® MALDI L™ linear time of flight mass spectrometer using:

- A nitrogen laser giving a 337 nm output of 3 ns pulse width.
- Laser fluence just above the threshold for ion production in the positive ion detection mode.
- An acceleration voltage of +15 kV.
- Automatic, accurate indexing of the sample/reference wells.
- Mass calibration using the average molecular weights from a (1:1) standard peptide mixture; (bradykinin, angiotensin I, glu-fibrinopeptide B, rennin substrate tetra decapeptide, ATCH (18-39 clip) all at 1 pmol/mL, bovine insulin 2 pmol/mL and ubiquitin 10 pmol/mL): matrix, α -cyano-4-hydroxycinnamic acid.
- A data acquisition mass range from m/z 500 to 10,000 Da.
- Automatic collection of bacterial mass fingerprints, and spectra from reference wells for lock mass calibration, using the MAXspec™ real-time data selection algorithm to optimize the bacterial fingerprint in the mass range 800-3000 Da.

Database construction

Each database entry consisted of a representative average spectrum derived from combining up to 12 replicate spectra for each bacterial strain. Significant outliers were eliminated using a root mean square (RMS) rejection value of 3.

RESULTS AND DISCUSSION

Bacillus species are ubiquitously disseminated in nature, being recovered from soil, dust, water, plants and animals. The broad range of physiological characteristics exhibited within the genus is reflected in the diverse range of species described. Some are capable of survival at extreme temperatures, pH or salinity thus species are encountered in artic soil to thermal springs and

from fresh water to marine sediments (2-4). The difficulties encountered in identification are largely due to the diverse metabolic activities of species and MALDI-TOF-MS was undertaken against a background of this immense heterogeneity.

Figure 3 shows examples of representative mass spectral profiles of five *Bacillus* species (*B. megaterium*, *B. laterosporus*, *B. coagulans*, *B. circulans* and *B. cereus*) and reveal the presence of distinct species biomarkers and therefore sufficient variability to delineate individual species.

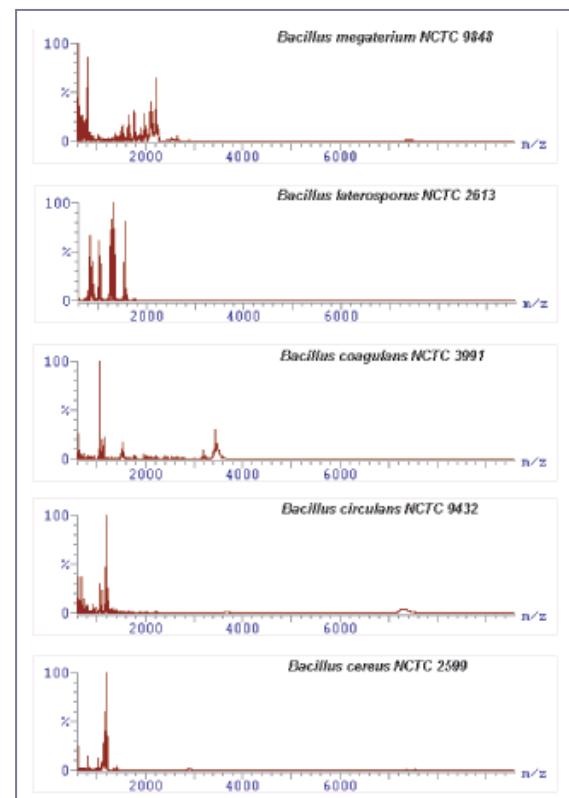


Figure 3. Mass ions produced from intact cells of five *Bacillus* species grown on Columbia Blood Agar. Cells were analyzed prior to sporulation.

Bacillus licheniformis contained the largest number of strains (16) tested so far in this study, consequently this species (represented by NCTC 8720) was used to search against the Database (2003 release) to assess the potential of the system. Figure 4 shows the confidence limit of this search which yielded a 99% relative probability and a RMS value of 2.36.

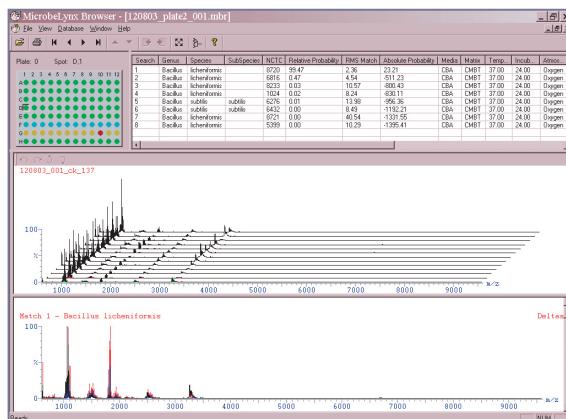


Figure 4. Database search results for *Bacillus licheniformis* NCTC 8720 indicating a high degree of confidence in these early results. It is evident from the work undertaken so far, that as the number of strains of each species is increased within the reference database, so does the accuracy of identification.

A cluster analysis using the Waters Masslynx™ and Microbelynx™ Software revealed significant differentiation of the 7 main *Bacillus* species so far studied here viz.

B. megaterium, *B. laterosporus*, *B. sphaericus*, *B. cereus*, *B. licheniformis*, *B. pumilus* and *B. subtilis*. (See Figure 5).

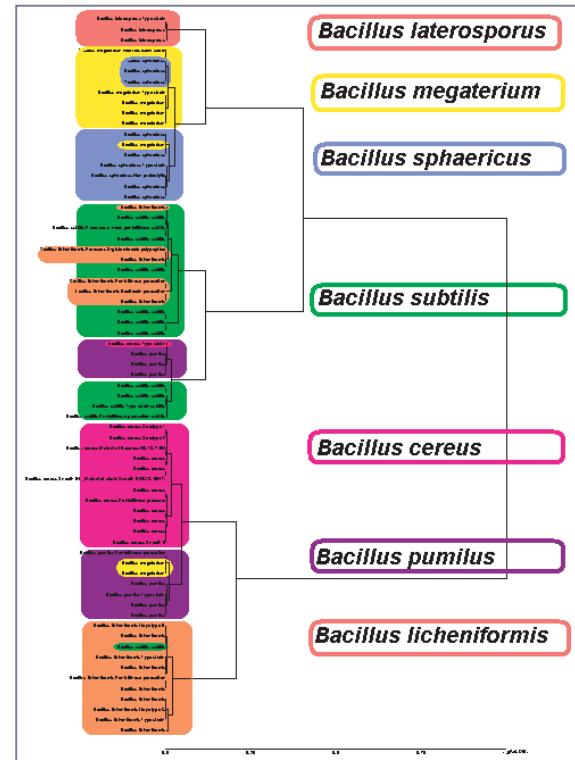


Figure 5. Cluster analysis using the Waters Masslynx™ and Microbelynx™ Software led to the recovery of most strains into clusters that, with few exceptions, paralleled their nomenclatural groups.

The results of these preliminary studies were sufficiently robust to warrant testing the system against wild type strains that were sent into the HPA for identification and were delineated by conventional methods. The results of this part of the study indicate that 70% of the isolates were identified correctly to the species level on the basis of the 1st match while 90% were identified correctly to species level when taking into consideration lower matches than the first. The most reliable data has so far been obtained for *B. licheniformis*, *B. cereus* and *B. subtilis*.

CONCLUSION

The results of this study indicate that the basic protocol established for these studies yielded a high degree of confidence in the data. However, the method utilizes intact cells, some of which are likely to remain viable during the laser ionization process. Several methods are currently being investigated to inactivate various bacilli prior to mass spectrometry. Preliminary data indicate that this has been achieved. Risk assessments are now being carried out in preparation for the imminent analysis of *Bacillus anthracis* using the above procedures.

REFERENCES

1. Farrar W.E. & Reboli A.C., 1991, The Genus *Bacillus*-Medical pp. 1746-1768. In M. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer (ed). The Prokaryotes; A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications (2 ed). Vol 2. Springer-Verlag, New York.
2. Stahly D.P., Andrews R.E. & Yousten A.A., 1991, The Genus *Bacillus*-Insect Pathogens pp.1697-1745. In M. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer (ed). The Prokaryotes; A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications (2 ed). Vol 2. Springer-Verlag, New York.
3. Slepecky R.A. & Hemphill H.E., 1991, The Genus *Bacillus*-Nonmedical. pp. 1663-1696. In M. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer (ed). The Prokaryotes; A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications (2 ed). Vol 2. Springer-Verlag, New York.
4. Claus D., Fritze D. & Kocur M. 1991, Genera Related to the Genus *Bacillus*-*Sporolactobacillus*, *Sporosarcina*, *Planococcus*, *Filibacter*, and *Caryophanon*. pp. 1769-1791. In M. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer (ed). The Prokaryotes; A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications (2 ed). Vol 2. Springer-Verlag, New York.
5. Anonymous. National Collection of Type Cultures and Pathogenic Fungi, 'Revival of Cultures: Opening of Ampoules': www.PHLS.org.uk/Labservices/nctc/revival.htm, 28/5/03
6. Claydon, M. A., Davey S N, Edwards-Jones V., & Gordon D. B., 1996, The rapid identification of intact microorganisms using mass spectrometry, *Nature Biotech.*, 14,1584-1586.
7. Dare, D. J., Bright, J. J., Morgan, M. M., Edwards-Jones, V, Keys, C. J., Shah, H. N., McKenna M. & Lunt, M., 2001, Rapid Identification of Bacteria by Intact Cell Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry:Inter-laboratory Study, *101st General Meeting of the American Society for Microbiology*, Florida, USA.
8. Dare, D.J., Bright, J.J., Sutton, H.E., Keys, C.J., Shah, H. N., McKenna, T., Lunt, M., & Wells, G., 2002, Rapid Identification of Bacteria by Intact Cell Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry, *102nd General Meeting of the American Society for Microbiology*, Salt Lake City, Utah, USA.
9. Sutton, H., E., Dare D., J., Keys, C. J., Shah, H., McKenna, T., Lunt, M., Willetts, M., & Zongmin. Du, 2003, Development of a Database for the Rapid Identification of *Staphylococcus aureus* by Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry, *103rd General Meeting of the American Society for Microbiology*, Washington DC, USA.
10. Fleming, K. Dare, D. J., Sutton, H. E., Edwards-Jones, V., Keys, C., J., Shah, H., McKenna, T., Willetts, M., & Lunt, M., 2003, Rapid Identification of Intact Bacterial Cells from Clinical Urinary *Enterobacteriaceae* Using Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry, *103rd General Meeting of the American Society for Microbiology*, Washington DC, USA.
11. Dare, D.J., Sutton, H.E., Keys, C. J., Shah, H. N., Wells, G & McDowall, M. A, 2003, Optimisation of a database for rapid identification of intact bacterial cells of *Escherichia coli* by MALDI-TOF MS, *51st American Society of Mass Spectrometry Conference*, Montreal, Canada.

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